Factors which control Encystment in *Pleurotricha lanceolata*

A Dissertation

Submitted to the Board of University Studies
of the Johns Hopkins University
in conformity with the requirements
for the degree of

Doctor of Philosophy

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Amos Benkov Kuan-Chin Penn

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1. Introduction.

Encystment in protozoa, since it was first observed in 1769 by SAUSSURE, has attracted the attention of many biologists. The literature on this subject includes forty some papers and books. The center of interest of these works is focused on the question

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as to the function of encystment and the factors which control the process. Diverse results have been obtained and diverse conclusions have been drawn by different workers from investigations on different animals.

The conclusions arrived at by different investigators concerning the factors which control encystment in protozoa can be summarized as follows:

1. Abundant food is necessary for the encystment of *Vampyrella*, *Vahlkampfia*, *Amoeba*, *Dinoflagellates*, *Didinium*, *Blepharisma*, and *Polytomella*.

2. Deficiency of food has an inducing effect in the encystment of predacious ciliates, *Vorticella*, *Stylonychia*, *Podophrya*, *Didinium*, *Spathidium*, and *Gastrostyla*.

3. Neither abundance nor lack of food influences the encystment of *Colpoda*.

4. Excretion products induce the encystment of *Amoeba*, *Didinium*, and *Colpoda*, but they do not induce encystment in *Polytomella*.

5. Excretion products of *Paramecium* do not induce encystment in *Didinium*.

6. Drying of the culture media produces encystment of *Stylonychia*, *Didinium*, and *Vorticella*, but not of *Gastrostyla*.

7. Age of culture is positively correlated with the encystment of *Didinium*.

8. Hydrogen-ion concentration is an inducing factor for encystment of *Colpoda*, and is a non-inducing factor for encystment of *Vorticella*, *Polytomella*, *Didinium*, *Gastrostyla*, and some races of *Colpoda*.

9. Crowding causes encystment in *Colpoda*, *Amoeba*, and *Blepharisma*.

10. Oxygen deficiency causes encystment in *Vorticella*, but not in *Colpoda* and *Gastrostyla*.

11. Temperature has no inducing effect on the encystment of *Amoeba*, *Didinium*, *Polytomella*, and *Gastrostyla*.

12. Loss of power of assimilation causes encystment in *Amoeba*.

13. Number of generations since previous encystment or since conjugation has no influence on the encystment of *Didinium*.

Many of these conclusions seem to be contradictory. However since different investigators worked on different animals, differences in result are not entirely unexpected. Most of them worked on mass cultures; the conditions of their experiments were not very well controlled; consequently, sources of error were likely to exist. Some of the factors with which they were working were not care-

fully analyzed; very often they worked with several factors while they thought they were working with one; hence their interpretations of their results are sometimes erroneous. Most of the work was not done on a quantitative basis and many conclusions were drawn from only superficial observations, so they are not always reliable.

In view of these facts, the views given by previous investigators concerning the factors which control encystment should be revaluated and further evidence should be sought in order to obtain a better understanding of this subject. It was for this purpose that the present work was undertaken.

2. Material and Methods.

Pleurotricha lanceolata was chosen as a subject for the experiments herewith presented. The strain of *Pleurotricha lanceolata* used is identical with that used by MANWELL in his work on the nuclear phenomena of the species. His pleurotrichs were grown in hay infusion to which a little peptone was added. His culture method was apparently not very satisfactory, because the organisms so cultivated were much smaller than those growing under natural conditions. I found that *Pleurotricha lanceolata* grew large and fat in PRINGSHEIM solution which had been inoculated with *Chilomonas* and *Colpidium*. But I did not use this culture method, because PRINGSHEIM solution prevented encystment. Solutions of timothy hay, wheat, rice and rye were tried; it was found that a 0.2 % hay and 0.2 % rye solution is most satisfactory.

This hay-rye solution was prepared by boiling in a beaker for eight minutes one gram of hay and one gram of rye in 600 ml. of spring water. After it had been boiled and cooled, there was about 500 ml. solution. Then half of the rye grains were removed, leaving the other half with all the hay in the solution. This was then transferred to a battery-jar of one liter capacity and left for two days, in order to allow bacteria to grow. When the infusion was two days old and contained many bacteria, it was inoculated with *Colpidium*. After twenty-four hours there were numerous colpidia present in the infusion. This was then inoculated with *Pleurotricha lanceolata*. The pleurotrichs grew well and divided rapidly, averaging four times a day. From time to time rich cultures of colpidia raised separately were added to the jar as food supply. In this way the culture could be kept in a flourishing condition for four days, after which the organisms encysted rapidly. Cultures thus prepared are designated as "stock cultures".

During the earlier part of the investigation, tap water was used in making up culture solutions, but owing to its inconstancy spring water was substituted. Besides *Colpidium*, *Chilomonas* was also used as food for the pleurotrichs. It was eliminated later, owing to its failure to grow with *Colpidium* in fresh infusions.

The size of *Pleurotricha lanceolata* varies greatly with the quality and quantity of food present in the medium. In natural conditions where a large variety of smaller protozoa is available for food, the length varies from 105 to 295 microns. MANWELL obtained an average length of 165 microns by growing the organisms in hay infusion with peptone. Probably *Chilomonas* was present in his cultures, although he failed to mention it. In my cultures, the organisms attained lengths ranging from 160 to 280 microns, with a majority about 215 microns. Extraordinarily large individuals were obtained by feeding the pleurotrichs their own cysts. Individuals containing several cysts in the body often measured from 300 to 350 microns in length. Such gigantic individuals have never been recorded in literature. The greatest length recorded is 300 microns (KENT, 1881—1882).

Cultures of *Colpidium* were also raised in a 0.2 % hay and 0.2 % rye infusion prepared as described above. Infusions two days old were poured into glasses of about 200 ml. capacity, then one piece of hay and two grains of rye, taken from the culture, were added to each glass. Fifty milliliters of pure culture of *Colpidium* were then added to each glass. On the next day, the glasses usually contained numerous colpidia. In order to provide the pleurotrichs with abundant food, three such cultures were prepared every day.

The rate of encystment in *Pleurotricha lanceolata* is closely related with the amount of food material present in the body of the organisms. Therefore, it is essential to standardize the organisms in respect to their food content. For this purpose, "standard-cultures" were prepared. About half a glass of colpidia culture was poured into a clean glass. Fresh two-day old infusion was added to it until the glass was about four-fifths full. Then two pipettes of pleurotrichs were taken from the stock culture and added to the diluted culture infusion. The pleurotrichs multiplied rapidly and devoured most of the colpidia within a day. No food was given on the second day, but the pleurotrichs continued to divide. On the third day, little food was left in their bodies. Pleurotrichs in his condition were used in the following experiments.

Experiments on encystment of *Pleurotricha lanceolata* were made in small salt dishes of 3 ml. capacity. One milliliter of freshly prepared hay-rye infusion was put into each dish, then ten pleurotrichs were transferred from the standard culture to each dish with a small pipette. In experiments in which clean specimens were necessary, the organisms were washed with fresh infusion before they were transferred to the dishes. After one day the dishes were examined for cysts and active organisms. Every experiment was repeated at least three times.

In order to facilitate counting of specimens in the dishes a low power dissection binocular microscope was used. The bottom of the dish was entirely in the field of the microscope. The specimens in every dish were counted at least twice. If the countings did not agree, the specimens were counted three or four times, and so on until two identical numbers were obtained. These numbers were assumed to be correct.

The percentage of encystment was calculated by dividing the total number of cysts in each set of five dishes by the total number of specimens including both cysts and free living organisms.

3. The Effect of Various Factors on the Rate of Encystment.

a) Food.

By looking over the results of different investigators on the effect of food on encystment, it appears that with some organisms, the effect is correlated with the amount of food in the media in which they live and with others with the amount of food in the body. In the former the factor involved is environmental, while in the latter the factor is physiological. These factors may act simultaneously, or one may act independently of the other. Many investigators failed to make this distinction and their results are therefore difficult to interpret.

In ascertaining the effect of the amount of food in the medium on the rate of encystment, seven sets of five dishes were prepared. One milliliter of culture infusion (0.2 % hay and 0.2 % rye in spring water) was put into each dish of all except one set. No food was added to the dishes in the first set. One drop of culture containing colpidia was added to each dish of the second set; two drops to each of the third set; four drops to each of the fourth set; eight drops to each of the fifth set; and twelve drops to each of the sixth set. To each dish of the seventh set, into which no culture

infusion had been put, was added one milliliter of culture containing many colpidia.

Then 10 specimens of *Pleurotricha* which had been washed were put into each dish. The dishes were now put into a constant-temperature chamber, the temperature of which was kept at 27°—28° and left 24 hours. Then the specimens and cysts in each dish were counted and recorded. The results obtained are presented in table 1.

Table 1.

The Effect of the Amount of Food in the Medium on the Rate of Encystment.

Amount of food in each dish	No. of individuals found	No. of cysts found	Av. % of encystment	Amount of food in each dish	No. of individuals found	No. of cysts found	Av. % of encystment
No food	20	12		8 drops	54	12	
	19	7			64	16	
	25	14	45.95		39	8	19.73
	23	8			56	13	
	24	10			61	9	
1 drop	32	13		12 drops	62	12	
	30	8			53	4	
	24	6	31.04		60	8	15.28
	31	10			55	10	
	28	8			58	10	
2 drops	31	10		1 ml.	68	10	
	35	10			75	15	
	38	9	26.40		71	10	12.78
	38	8			66	2	
	36	10			72	8	
4 drops	52	8					
	47	11					
	51	12	18.93				
	45	6					
	48	9					

It will be observed from this table that the rate of encystment varied inversely with the amount of food present in the medium. This agrees with the results obtained by MAUPAS (1888) on predaceous ciliates, by ENRIQUES (1907) on *Vorticella*, by FERMOR (1913) on *Stylonychia*, by VON BRAND (1923) on *Vorticella*, by MOORE (1924) on *Spathidium*, by ROOT (1914) on *Suctoria*, by MAST (1917) and BEERS (1927) on *Didinium*, and by WEYER on *Gastrostyla* (1930) but not with the results obtained by HOGUE (1915) on *Vahlkampfia*; CARTER (1919), KOFOID and SWEZY (1921) on *Amoeba* and marine dinoflagellates and MAST and IBARA (1923) on *Didinium*.

The effect of the amount of food in the organism on the rate of encystment was ascertained as follows: Several hundred pleurotrichs were transferred from the stock culture to each of two small

beakers containing about 50 ml. freshly prepared hay-rye infusion. The beakers were then put into a constant-temperature chamber at 10^0 and left twenty-four hours. On account of lack of food and low temperature, the pleurotrichs were gradually starved. Only a few encysted. These cysts were all taken out, leaving only free-living pleurotrichs in the beakers. Then, to one beaker, 50 ml. of Colpidium culture was added as food; to the other, no food was added. After thirty minutes, the pleurotrichs in the beaker with colpidia contained many large food vacuoles while those in the beaker without food contained only a few small food vacuoles.

Two sets of five dishes, each dish containing 1 ml. of freshly prepared hay-rye infusion were prepared. To each dish of one set, ten starved pleurotrichs were added, and to each of the other set, ten wellfed pleurotrichs. Both sets were put into the constant-temperature chamber at 26^0—27^0. After 24 hours, the dishes were examined for cysts and free living individuals. The results obtained are given in table 2.

Table 2.

The Effect of the Amount of Food Material in the Body of the Organism on the Rate of Encystment.

Organisms starved for 24 hours			Organisms well fed		
No. of individuals found	No. of cysts found	Av. % of encystment	No. of individuals found	No. of cysts found	Av. % of encystment
12	7		48	2	
12	9		51	8	
14	10	63.08	42	0	7.11
13	8		44	2	
14	7		40	4	

This table shows that the organisms with less food material in their body encysted much more readily than those with more. This proves that starvation is an inducing factor in encystment.

This result is just opposite to the results obtained by Kepner (1924) on *Vampyrella*, by Stolte (1922) on *Blepharisma* and by Kater und Burroughs (1926) on *Polytomella*.

This diversity of results is probably due to difference in function of encystment in different organisms. In organisms in which encystment serves to bridge over adverse environmental conditions, lack of food should facilitate encystment, whereas in those in which it functions in digestion and in reproduction food should facilitate encystment.

b) Temperature.

Eight different temperatures were studied: 9°—10°, 18°—19°, 20°—21°, 23°—24°, 26°—27°, 29°—30°, 34°—35°, 38°—40°. The six lower temperatures were obtained in different compartments of a graded constant-temperature chamber, the remaining two in two separate thermostats. Eight sets of five dishes, each dish containing 1 ml. of freshly prepared hay-rye infusion were prepared. Ten pleurotrichs taken from the standard culture were added to each dish. Then one of the sets was put into each temperature compartment. After 24 hours the dishes were examined for cysts and free living individuals. The results obtained are presented in Table 3.

Table 3.

The Effect of Various Temperatures on the Rate of Encystment.

Temperature	No. of individuals found	No. of cysts found	Av. % of encystment	Temperature	No. of individuals found	No. of cysts found	Av. % of encystment
9°—10°	11	0	No encystment	26°—27°	35	19	58.78
	10	0			33	24	
	10	0			32	22	
	12	0			36	17	
	11	0			29	15	
18°—19°	23	0	1.00	29°—30°	25	10	51.85
	19	0			20	12	
	22	1			23	12	
	16	0			22	9	
	20	0			18	13	
20°—21°	18	0	3.48	34°—35°	11	5	34.00
	14	2			12	3	
	23	0			8	3	
	27	0			9	4	
	23	2			10	2	
23°—24°	31	9	33.13	38°—40°	0	0	0
	34	10			0	0	
	30	13			0	0	
	36	12			0	0	
	35	11			0	0	

It should be said that at 34°—35° some of the pleurotrichs disintegrated, others became deformed, still others seemed to have begun encystment but did not complete it, and that at 38°—40° all the pleurotrichs disintegrated.

The table shows that as the temperature increased from 9° the rate of encystment and the rate of fission increased, at first slowly and then very rapidly to a maximum at 26° -27° and then decreased. This indicates that encystment is facilitated by temperature which

is favorable for growth. This is in accord with the results of Mast and Ibara (1923) on *Didinium* and of Kater and Burroughs (1926) on *Polytomella* and of Weyer on *Gastrostyla* (1930). This correlation should however not be overemphasized, for at 23°—24° and 26° —27° the rates of fission were nearly the same, and yet the rate of encystment was much lower in the former than in the latter, and in all lower temperatures the rate of encystment was relatively much lower than the rate of fission.

Table 3 shows that no encystment occurred in the temperature 9°—10°. The question now arises as to whether low temperature actually prevents or merely retards the process of encystment. An attempt was made to answer this question as follows:

To each of a set of five dishes, containing 1 ml. of freshly prepared hay-rye infusion, 10 pleurotrichs were transferred from the standard culture. Then all the dishes were put into the constant-temperature chamber at 9° —10°. Examinations for cysts and free living individuals were made daily for five days and also once on the eighth day. The results are presented in table 4.

Table 4.

The Effect of Low Temperature (9°—10°) on the Rate of Encystment.

Date	No. of individuals found	No. of cysts found	Av. % of encystment	Date	No. of individuals found	No. of cysts found	Av. % of encystment
1 st day	12	0		4 th day	26	1	
	11	0			24	3	
	14	0	No encystment		24	0	6.09
	12	0			20	3	
	12	0			21	1	
2 nd day	18	0		5 th day	29	3	
	20	0			27	4	
	19	0	No encystment		26	2	12.21
	16	0			24	4	
	18	0			25	3	
3 rd day	20	0		8 th day	41	19	
	21	1			37	12	
	21	0	1.01		38	15	41.62
	18	0			33	16	
	19	0			36	15	

This table shows that during the first two days, no encystment occurred, that on the third day cysts were found, and that on the eighth day, the percentage of encystment was considerably higher. This indicates that low temperature does not prevent encystment, but merely retards it. It is therefore not in harmony

with Kater and Burroughs's conclusion (1926) that low temperature prevents encystment. Furthermore, all former workers spoke of unfavorable temperatures, both high and low, as though the effect on encystment were the same. My experiments show that the effects produced by high and low unfavorable temperatures are different in nature. They show that low temperature merely retards encystment as it does many other physiological processes, and is not injurious, while unfavorable high temperatures retard encystment owing to injury.

c) Acidity and Alkalinity.

The effect of acidity and alkalinity on the rate of encystment was ascertained as follows: Rye infusion (0.4%) was used in place of hay-rye infusion. It was prepared in the same way as the hay-rye infusion. The hay was eliminated because it usually produces a yellowish color which interferes with the measurement of hydrogen-ion concentration by the colorimetric methods used.

Eleven solutions ranging in hydrogen-ion concentration from p_H 4 to p_H 9 were made by adding to the rye infusion N/100 HCl or NaOH. One ml. of one of these solutions was put into each dish in eleven sets of 5 square salt dishes, one solution to each set, making 11 sets, each set containing solution of a specific acidity. Then 10 pleurotrichs from the standard culture were cleaned carefully and added to each dish, after which the dishes were put into the constant-temperature chamber at 22°—23° and left 24 hours. At the end of that period, examinations for cysts and free-living individuals were made. The results are presented in table 5 (see p. 13).

From this table we see that, as the hydrogen-ion concentration decreased from p_H 4.0, the rate of encystment increased to a maximum at about p_H 5.5 and then remained fairly constant until p_H 8.5 was reached, after which it declined. This indicates that encystment is independent of hydrogen-ion concentration over a surprisingly wide range. In infusions of extreme acidity and alkalinity, the lowering of the rate of encystment is probably not the direct consequence of the action of the hydrogen-ion or hydroxyl-ion concentration on encystment, but the consequence of unfavorable abnormal physiological change. Similar results were obtained by van Brand (1923) on *Vorticella*, by Kater and Burroughs (1926) on *Polytomella*, by Barker and Taylor (1931) on *Colpoda*, and by Beers on *Didinium* (1930) and by Weyer on *Gastrostyla* (1930). Diverse results were obtained by Koffman (1924) on certain ciliates and by Darby (1929) on

Table 5.

The Effect of Acidity and Alkalinity on the Rate of Encystment.

p_H	No. of individuals found	No. of cysts found	Av. % of encystment	p_H	No. of individuals found	No. of cysts found	Av. % of encystment
4.0	31	0	2.68	7.0	31	4	40.00
	26	1			31	16	
	29	1			29	9	
	29	0			36	21	
	34	2			33	14	
4.5	20	4	8.00	7.5	32	18	38.16
	35	3			35	11	
	36	3			25	6	
	28	0			29	12	
	31	2			31	11	
5.0	28	9	32.41	8.0	30	15	40.27
	30	15			30	12	
	31	7			32	10	
	30	11			28	14	
	26	5			29	9	
5.5	32	14	42.38	8.5	28	6	35.15
	35	12			33	13	
	27	8			29	16	
	27	11			31	11	
	30	9			34	12	
6.0	36	19	39.35	9.0	29	6	30.26
	28	9			29	8	
	31	8			29	5	
	32	15			34	13	
	28	10			31	14	
6.5	28	14	41.72				
	28	6					
	32	18					
	34	17					
	29	8					

mixed cultures of protozoa. These are not to be seriously considered, because they were obtained in mass cultures where many other factors were involved.

d) Age of the Hay-rye Infusion.

On each day, for a period of eight days, 50 ml. of hay-rye infusion prepared as described above, was transferred to a large vial with one grain of rye and one piece of hay. The vials were placed side by side in the laboratory in diffuse sunlight. Thus infusions differing in age from one day to eight days were obtained. One ml. of each of these infusions was put respectively into each of five square salt dishes.

Eight sets of five dishes each were prepared. To each dish of the first set 1 ml. of 1-day old hay-rye infusion was added; to each

of the second set 1 ml. of 2-day old infusion; to each of the third set 1 ml. of 3-day old infusion; to each of the fourth set 1 ml. of 4-day old infusion; to each of the fifth set 1 ml. of 5-day old infusion; to each of the sixth set 1 ml. of 6-day old infusion; to each of the seventh set 1 ml. of 7-day old infusion; and to each of the eighth set 1 ml. of 8-day old infusion. Then 10 pleurotrichs from the standard culture were added to each dish. The dishes were now put into a constant temperature chamber at 22°—23°, left 24 hours and then examined for cysts and free-living individuals. The results obtained are given in table 6.

Table 6.

The Effect of the Age of Hay-Rye Infusion on the Rate of Encystment in *Pleurotricha*.

Age of hay-rye infusion	No. of individuals found	No. of cysts found	Av. % of encystment	Age of hay-rye infusion	No. of individuals found	No. of cysts found	Av. % of encystment
1 day	33	13		5 days	24	19	
	36	15			26	22	
	34	12	39.77		21	18	83.06
	37	17			28	20	
	31	11			25	24	
2 days	32	18		6 days	21	11	
	35	16			23	14	
	30	13	48.46		18	7	55.66
	36	17			20	12	
	30	15			24	15	
3 days	35	20		7 days	25	14	
	34	18			16	9	
	32	24	63.74		21	10	57.94
	37	25			24	11	
	33	22			21	8	
4 days	28	18		8 days	22	8	
	31	23			22	11	
	25	19	71.43		18	11	46.23
	29	21			21	9	
	33	24			23	10	

This table indicates the following: As the age of the infusion increases, the rate of encystment increases gradually to a maximum in the five-day old infusion and then decreases irregularly. The decrease in the rate of encystment does not seem to follow any regular order. It appears that it is independent of the age of infusion beyond five days old. However, in infusions 6-days, 7-days and 8-days old, it is generally higher than in infusions 1-day and 2-days old. The rate of fission is generally higher in younger infusions than in older ones. This indicates that the older infusions are unfavorable for growth. This is in harmony with the results

obtained by Beers (1927) on *Didinium*, except that he did not obtain cysts in infusions one and two days old. The effect of the age of infusion on encystment is apparently due to the chemical changes of the infusion as a result of inter-action of substances and activities of bacteria in the solution.

e) Excretion Products of Different Organisms.

In the following three experiments attemps were made to solve the subsequent questions:

1. Do the excretion products of a given organism facilitate the process of encystment in the organism?

2. Do the excretion products of the organism used as food inhibit encystment of the organism?

3. Do the excretion products of all organisms facilitate or inhibit encystment in all organisms?

1. *Pleurotricha.*

Thousands of pleurotricha, taken from the stock culture, were with a centrifuge washed four times in freshly prepared hay-rye infusion so as to obtain many specimens practically free from small particles and other organisms which occur in the stock culture solution. These pleurotrichs were then concentrated by centrifuging and transferred to a small beaker containing 50 ml. of freshly prepared hay-rye infusion which had been filtered so as to remove all particles. The beaker was put into a constant temperature chamber at 18°—19° and left two days, allowing excretion products to accumulate. Then all the pleurotrichs and cysts in the beaker were removed by filtration, and a clear solution containing excretion products was obtained.

Another small beaker containing 50 ml. of freshly prepared hay-rye infusion was treated in precisely the same way, except that no pleurotrichs were added. Consequently no excretion products of pleurotrichs were produced in this infusion.

To each of a set of five dishes 1 ml. of pure hay-rye infusion was added; to each of a second set 1 ml. of the solution containing excretion products of *Pleurotricha;* and to each of a third set $^1/_2$ ml. of pure hay-rye infusion and $^1/_2$ ml. of the solution with excretion products of *Pleurotricha.* Then 10 pleurotrichs from the standard culture were added to each dish, after which all the dishes were put into a constant-temperature chamber at 22°—23°, left 24 hours and then examined for cysts and free-living individuals. The results are given in table 7.

Table 7.

The Effect of Excretion Products of *Pleurotricha* on the Rate of Encystment of *Pleurotricha*.

Medium	No. of individuals found	No. of cysts found	Average percentage of encystment
Hay-rye infusion	32	14	
	33	6	
	27	12	32.05
	35	14	
	29	4	
Half hay-rye infusion, half solution with excretion products of *Pleurotricha*	28	17	
	25	11	
	26	18	55.91
	26	15	
	22	10	
Solution with excretion products of *Pleurotricha*	8	8	
	10	9	
	6	5	89.19
	4	4	
	9	7	

This table clearly indicates that the higher the concentration of excretion products of *Pleurotricha* the higher the percentage of encystment. It is therefore evident that the rate of encystment of *Pleurotricha* is greatly facilitated by its own excretion products. This is in accord with results obtained by HOGUE (1915) and MAST and IBARA (1929) on *Amoeba*, *Didinium* and *Colpoda*, but not with those obtained by KATER and BURROUGHS (1926) on *Polytomella*.

In the solution containing the highest concentration of excretion products some of the organisms disintegrated. This disintegration was probably due to the injurious effect of the high concentration of the excretion products.

2. *Colpidium*.

Debris was removed from a dense culture of *Colpidium* by decantation. The colpidia were then washed with a centrifuge four times in freshly prepared hay-rye infusion, transferred to a small beaker and left two days, allowing excretion products of *Colpidium* to accumulate. At the same time, an equal amount of freshly prepared hay-rye infusion was poured into another small beaker and left two days. The colpidium culture was then filtered three times to remove all colpidia. The hay-rye infusion was also filtered three times to remove all particles present.

To each of a set of five dishes 1 ml. of the hay-rye infusion was now added; to each of a second set, 1 ml. of the solution with excretion products of *Colpidium*; to each of a third set $^1/_2$ ml. of the hay-rye infusion and $^1/_2$ ml. of the solution with excretion products of *Colpidium*. Then to each dish ten pleurotrichs taken from the standard culture were added, and all the dishes put into a constant-temperature chamber at 22°—23°, left 24 hours and then examined for cysts and free-living individuals. The results are given in table 8.

Table 8.

The Effect of Excretion Products of *Colpidium* on the Rate of Encystment of *Pleurotricha*.

Medium	No. of individuals found	No. of cysts found	Average percentage of encystment
Hay-rye infusion	34	9	
	30	6	
	36	15	31.98
	32	11	
	40	14	
Half hay-rye infusion; half solution with excretion products of *Colpidium*	24	15	
	29	18	
	28	11	53.44
	23	12	
	27	14	
Solution with excretion products of *Colpidium*	13	12	
	14	12	
	12	10	90.48
	11	11	
	13	12	

This table shows that the percentage of encystment in the solutions containing excretion products of *Colpidium* was decidedly higher than that in those containing no excretion products. This clearly indicates that excretion products of *Colpidium*, an organism used as food by *Pleurotricha*, facilitates encystment in *Pleurotricha*. This is not in harmony with conclusions reached by Beers (1927) in his observations on encystment in *Didinium*.

3. *Paramecium*.

A two-day-old hay-rye infusion was inoculated with *Paramecium*. Five days after inoculation the paramecium culture reached a flourishing condition. Thousands of paramecia were taken from the culture and washed three times in freshly prepared hay-rye infusion with a centrifuge to remove all particles and other smaller organisms.

The cleaned paramecia were transferred to a beaker containing freshly prepared hay-rye infusion which had been filtered. The beaker was then left two days, allowing excretion products of *Paramecium* to accumulate. At the same time, an equal amount of filtered freshly prepared hay-rye infusion was poured into another beaker and placed side by side with the solution containing paramecia. The solution in the two beakers was filtered to remove the paramecia from the one and particles from the other.

To each of a set of five dishes, 1 ml. of hay-rye infusion was now added; to each of a second set, 1 ml. of solution with excretion products of *Paramecium*; to each of a third set, $^1/_2$ ml. of hay-rye infusion and $^1/_2$ ml. of solution with excretion products of *Paramecium*. Ten pleurotrichs from the standard culture were then added to each dish of every set after which the dishes were put into a constant-temperature chamber at 22°—23°, left 24 hours, and then examined for cysts and free living individuals. The results are presented in table 9.

Table 9.

The Effect of Excretion Products of *Paramecium* on the Rate of Encystment in *Pleurotricha*.

Medium	No. of individuals found	No. of cysts found	Average percentage of encystment
Hay-rye infusion	30	3	
	28	3	
	35	8	20.78
	32	13	
	29	5	
Half hay-rye infusion; half solution with excretion products of *Paramecium*	23	15	
	28	16	
	26	9	52.85
	21	11	
	25	14	
Solution with excretion products of *Paramecium*	12	12	
	11	11	
	14	13	95.10
	12	12	
	12	10	

This table indicates that the rate of encystment in hay-rye infusion is low, that it increases when excretion products of *Paramecium* are added, and that it becomes very high when these excretion products are concentrated. By comparing tables 7, 8 and 9 it will be seen that the excretion products of *Paramecium* are just as efficient as those of *Pleurotricha* and *Colpidium* in inducing encystment in *Pleurotricha*.

f) Bacteria.

It is well known that bacteria play a very important rôle in the life of protozoa; they serve as food for many protozoa; they act on organic substances present in the medium and thereby change its chemical composition; and they give off excretion products. It is therefore evident that the results obtained in the preceding experiments may have been greatly influenced by the action of the bacteria present in all of them, and that it is consequently highly desirable to have them repeated with solutions in which the bacteria are controlled.

In order to perform experiments under bacteriological control, a method for biological sterilization of *Pleurotricha* is necessary. The pipette method of Glaser and Coria (1930) was tried without success. Then the transferring method, devised by Hargitt and Fray (1917) and modified by Parpart (1928) was tried. Since the pleurotrichs are large enough to be plainly visible to the naked eye and since the experiments were performed in a quiet room, the microscope and the hood used by Parpart were not necessary. Otherwise, the technique of Parpart was followed with only a few necessary modifications as to the available glassware, medium, and intervals between washings. Although this method proved successful, it was far from practicable. For, in each experiment, a large number of sterile animals was needed. To sterilize ten individuals at a time, more than one hundred Petri-dishes and an equal number of depression slides would be required. To handle such a bulk of apparatus, and to carry out delicate processes of sterilization with them, was practically impossible.

Faced with such difficulty, Parpart's method was modified so as to make it practicable. The lines of modification were directed towards increase in distance of migration so as to increase the efficiency of washing, decrease in number of transfers and reduction in the amount of necessary apparatus. The following satisfactory technique was devised:

1. *Pleurotricha* was washed nine times. The first three and the seventh washings were performed directly in Petri-dishes 95 mm. in diameter. The fourth, fifth, sixth, and the last two washings were performed in watch glasses which were in Petri-dishes.

2. The pipettes used in transferring the animals were made from glass tubing having an inner diameter of 6 mm, and a wall of 1 mm thickness. One end of the tubing was drawn out to

capillary fineness with a tapering part about 30 mm long. This end was fitted with a plug of cotton into a cap, made from glass tubing of larger size, while the other end was plugged with cotton and was temporarily attached to a rubber bulb when handling animals.

3. The pipettes, watch glasses, and PETRI-dishes were sterilized in a dry air sterilizer at 110°—120° for one hour or more, except once a week when an autoclave was used.

4. The extract used as the washing medium was prepared by boiling for ten minutes, ten grains of rye (about 400 mgm in weight) in each 100 ml. of distilled water. This rye extract was poured into 250 ml. flasks which were plugged with cotton and then sterilized in an autoclave at 10—16½ lb. pressure for at least 15 minutes.

5. Before starting the processes of washing, the PETRI-dishes were spread on the table, then the empty ones were filled half full with rye extract, and to each watch glass in the others was added 1 ml. of rye extract. The glassware and the medium were cooled to room temperature beforehand, so that no moisture condensed in the PETRI-dishes to obscure vision.

6. As stated above, the first three washings were performed directly in PETRI-dishes. About ¼ ml. of concentrated *Pleurotricha* culture containing hundreds of individuals were transferred to the first PETRI-dish at the left edge by lifting the cover just enough to permit the entrance of the transferring pipette. As the animals were very active, a large number of them by random migration reached the right edge of the dish within two minutes. The ones first to arrive (about 50 in number) were quickly picked up and transferred to the second dish also at the left edge. Some of them swam across to the right edge of the dish, and the first ten to arrive were transferred to a third dish and left in it for half an hour.

As the animals swam across the first and the second dishes, most of the bacteria adhering to the body of the animals were washed off. They were left in the third dish for half an hour in order to give ample time for bacteria to leave them.

From the third dish, each animal was transferred to rye extract in a watch glass, in a PETRI-dish. This constituted the fourth washing. At ten minute intervals, the animal was carried through successively from the fourth to the fifth and from the fifth to the sixth washings. In the sixth washing medium, the animal was left for at least five hours (often over night) to allow the spores of spore formers, to become active.

From the sixth washing, the animal was again transferred to a PETRI-dish and washed in the same way as in the second PETRI-

dish. Then it was carried through the eighth and nineth washings in the same way as through the fourth and the fifth.

Petri-dishes were used in the first three and the seventh washings instead of watch glasses in Petri-dishes in order to increase the distance of migration and the efficiency of washing. As the washing medium was in direct contact with the Petri dishes, the edges of these dishes had to be carefully guarded against bacteria. By using freshly sterilized dishes and by handling them with great care, bacterial infection from the edges was avoided.

Tests by standard nutrient agar plate for bacterial infection of washing media showed that the medium of the fifth washing was in most instances sterile, that that of the sixth was usually sterile when tested within an interval of ten minutes, sometimes infected when tested after the animal was there for a longer time, that the medium of the eighth washing was usually sterile, and that that of the nineth was always sterile.

Three experiments were performed, the first to test the effect of bacteria as a whole, the second to test the effect of bacterial activity including excretion products of bacteria, and the third to test the effect of absence of bacteria.

The medium used in these and the following experiments was prepared by boiling 0.4 gm. of rye in each 100 ml. of distilled water for ten minutes. This will be referred to as "standard rye extract".

Experiment 1.

Two Erlenmyer flasks, each containing 200 ml. of standard rye extract were plugged and autoclaved. After they had been cooled to room temperature, one of them was inoculated with bacteria from a pleurotricha culture, and the other left sterile. The flasks were then set away for the bacteria to multiply in the inoculated flask. After six days, 1 ml. of solution was transferred from each of the two flasks to Columbia dishes in Petri dishes. Then ten sterile pleurotrichs were put into each Columbia dish. These were now put into a constant-temperature chamber at 23°—24°, left 24 hours, then cysts and free-living individuals were counted and recorded in table 10a (see p. 22).

It will be seen by referring to this table, that the rate of encystment was lower in the solution with bacteria than in that without bacteria. Close examination of the organism revealed that those in the solution with bacteria were large and fat and appeared white,

Table 10.

The Effect of *Bacteria* on the Rate of Encystment of *Pleurotricha*.

Solution	No. of individuals found	Number of cysts found	Average percentage of encystment
	a		
6-day-old rye extract with bacteria	19	2	11.90
	15	3	
	18	3	
	17	0	
	15	2	
6-day-old sterile rye extract	15	8	24.69
	17	5	
	14	4	
	19	4	
	19	6	
	b		
4-day-old rye extract with bacteria removed	16	8	55.26
	17	5	
	13	10	
	15	12	
	17	7	
4-day-sterile rye extract	15	6	22.62
	19	4	
	18	2	
	18	2	
	14	5	
	c		
1-day-old sterile rye extract	22	5	35.12
	25	8	
	19	2	
	30	13	
	24	10	
	24	7	
	21	8	
	25	12	
	29	11	
	23	9	
1-day-old rye extract with bacteria	24	6	37.55
	21	9	
	23	5	
	28	11	
	22	12	
	28	9	
	23	10	
	24	9	
	27	13	
	25	8	

while, those in the solution without bacteria were smaller and thin and appeared more opaque. This shows that bacteria had been used as food. This probably caused the difference in the rate of encystment.

Experiment 2.

This experiment was performed in the same way as Experiment 1, except that the rye extract, which had been inoculated with bacteria and allowed to stay for four days, was filtered through a Zeiss filter to remove all bacteria, leaving only the soluble excretion products of bacteria in the solution. The Zeiss filter was cleaned first by boiling for half an hour in a large amount of water, then by placing it in running water for two hours, and finally by repeated autoclaving in water. This filter was fitted into a suction flask by a rubber stopper. The arm of the suction flask was plugged with cotton and connected with a suction pump by a piece of rubber tubing. A new asbestos filter pad was used for each filtration. The filtrate was sterile as indicated by inoculation of agar plates. The sterile solution in the control was treated exactly the same way so as to eliminate any possible error due to filtration. The results obtained are presented in 10 b.

From this table it will be seen that in the solution in which bacterial activity had been continued for four days but from which the bacteria had been removed by filtration, the rate of encystment was much higher than that in the sterile four-day-old solution.

Experiment 3.

This experiment consists of two sets just as in the preceding experiment: one with bacteriological control, the other without.

All conditions were the same in both sets, except that the later set was exposed to infection of bacteria from glassware, from the animals, and from the air. Both sets were put into a constant-temperature chamber at 23°—24°, left 24 hours, and then examined for cysts and active pleurotrichs. The results are recorded in table 10 c.

It will be observed from this table that the rate of encystment was practically the same in both sets. The slight difference in rate is not considered significant, because such small difference is well within the range of variation.

It is evident then, that the rate of encystment in the preceding experiments performed without bacteriological control were but little if at all affected by the presence of bacteria, and that the conclusions reached in the preceding sections are therefore valid.

g) "Crowding".

"Crowding" has been reported to induce encystment of *Colpodia*, *Amoeba*, and *Blepharisma* by Hogue (1915), Stolte (1922) and Barker and Taylor (1931). Working on mass cultures, these investigators

noticed that when cultures became crowded, encystment occurred. During the course of the present investigation and in preliminary studies on *Bursaria* and *Nassula*, the same phenomenon was observed. Now, why does crowding cause encystment? Neither Hogue, nor Stolte, nor Barker and Taylor gave any adequate explanation. In order to answer this question, the following experiment was designed:

This experiment was carried out in watch-glasses each in a Petri-dish. Throughout the whole experiment, the bacteriological technique discribed above was used. Three sets of tests, A, B and C were made. In set A, a single individual of *Pleurotricha* was put into 1 ml. of rye extract in each of ten watch-glasses, each in a Petri-dish, and in set B, ten individuals were put into the same amount of rye extract in one watch-glass in a Petri-dish. The Petri-dishes were then put into a constant-temperature chamber at 23°—24°. After twenty-four hours, examination for cysts and free living individuals was made. Then, the free living animals were transferred to fresh rye extract and left in the constant-temperature chamber for another 24 hours, after which examinations were again made.

In set C ten pleurotrichs were put into 1 ml. of rye extract in a watch-glass in a Petri-dish. This was put into a constant-temperature chamber at 23°—24°, and left 24 hours. Then the active animals and the cysts were removed from the medium and a freshly prepared individual like that used in set A was introduced, after which the dish was again put into the constant-temperature chamber at 23°—24°. After 24 hours, the cysts and free-living organisms were counted and the numbers recorded. These three tests were repeated several times. The results are presented in table 11 (see p. 25).

Table 11 shows that with one pleurotrich in 1 ml. of sterile rye extract the rate of encystment was very much lower than with ten in the same amount of the solution, and that with excretion products added to the sterile rye extract the rate of encystment was much higher than it was without these products added. This shows that crowding induces encystment and it indicates that the effect of crowding is due to the accumulation of waste products, and is therefore not directly correlated with it.

h) Urine.

Experiments in sub-sections e, f, and g, show that excretion products of *Pleurotricha*, *Colpidium*, *Paramecium*, and *Bacteria* induce encystment. Now the question arises: What substance or

Table 11.

The Effect of "Crowding" on the Rate of Encystment.

First day				Second day			
Initial number of animals	No. of individuals found	No. of cysts found	Av. % of encystment	Initial number of animals	No. of individuals found	No. of cysts found	Av. % of encystment
a) One animal in one ml. sterile rye extract							
41	94	1	1.03	41	86	38	43.18
b) Ten animals in one ml. sterile rye extract							
70	150	59	39.33	64	109	71	65.14
c) One animal in one ml. sterile rye extract + excretion products of *Pleurotricha*							
10	22	7	31.82				

substances in the excretion products are responsible for inducing encystment? A conclusive answer to this question requires an extensive investigation concerning the constituents of the excretion products of these animals. Our present limited knowledge about the excretion products of protozoa, renders such an attempt very difficult. However, it is of interest to ascertain the effect of the essential components of excretion products of higher animals on encystment. Among the substances excreted by higher animals, urine is very prominent. Does urine affect encystment?

Solutions with different concentrations of urine were prepared by diluting freshly excreted urine with standard rye extract. To each of a set of five salt dishes 1 ml. of solution containing 10% urine was added; to each of a second set, 1 ml. of 1% urine; to each of a third set, 1 ml. of 0,1 % urine; to each of a fourth set, 1 ml. of 0.01% urine; to each of a fifth set, 1 ml. of standard rye extract with no urine. Then into each dish ten thoroughly washed pleurotrichs from a standard culture were put. The dishes were covered and put into a constant-temperature chamber at 23°—24°. After 24 hours the cysts and free-living individuals were counted and the numbers recorded in table 12 (see p. 26).

This experiment was repeated with sterile and without sterile solutions. The results obtained are similar. In experiments with sterile solutions bacteria free pleurotrichs and pasteurized urine were used.

This table shows that in 10% urine all the animals desintegrated that in 1% no encystment occurrred; that in 0.1% urine cysts

Table 12.

The Effect of Urine on the Rate of Encystment.

Concentration of urine %	Number of individuals found	Number of cysts found	Average percentage of encystment
10	0	0	
	0	0	
	0	0	0
	0	0	
	0	0	
1	20	0	
	23	0	
	18	0	0
	24	0	
	23	0	
0.1	19	0	
	25	0	
	17	2	2.94
	18	1	
	23	0	
0.01	21	3	
	20	0	
	21	2	11.11
	18	2	
	19	4	
Control No urine	18	4	
	22	2	
	17	7	16.67
	18	0	
	21	3	

were occasionally found; and that in 0.01 % urine the percentage of encystment was decidedly lower than in the control.

It was observed that in the solutions containing the higher concentration of urine, the rate of fission was slightly higher than that in rye extract without urine. The animals looked fat and retained their size after division. This probably indicates that the animals were able to use urine as nutrient, or else that the urea in the urine caused an increase in permeability of the animal so that more nutritive material from the rye extract passed into the cell.

i) Ammonia.

Ammonia is among one of the important excretion products of higher animals. It has also been found in the excretion products of some protozoa. Its probable effect on encystment has been discussed by a number of investigators, but no test has been made.

In order to ascertain the effect of ammonia, the following experiment was performed. The procedure was like that described

above. Three concentrations of ammonium chloride (0.1 M, 0.01 M, and 0.001 M), were used.

This experiment was repeated with sterile and without sterile solutions. The results obtained are practically alike. They are presented in table 13.

Table 13.

The Effect of NH_4Cl on the Rate of Encystment.

Concentration of NH_4Cl	Number of individuals found	Number of cysts found	Average percentage of encystment
0.1 M	0	0	
	6	0	
	0	0	0
	0	0	
	2	0	
0.01 M	14	0	
	11	0	
	13	0	0
	11	0	
	9	0	
0.001 M	21	7	
	18	0	
	22	5	22.12
	20	8	
	23	3	
Control No NH_4Cl	17	10	
	24	8	
	22	11	44.55
	26	6	
	21	14	

This table shows that the rate of encystment in the control was higher than that in any of the solutions containing ammonium salt.

When the concentration of ammonium chloride reaches 0.1 M, the solution becomes very toxic and disintegration of the animal results. In the lower concentrations the animals appear normal, but very few of them encyst. This indicates that ammonia does not induce encystment, but on the contrary inhibits it.

j) Salts.

Salts are present in excretion products and in all kinds of media. They play a very important rôle in the life of protozoa. A disturbance in the balance of salts often leads to serious results. Practically all physiological processes are directly or indirectly correlated with the kind and the concentration of salts present.

Hence, it seems important that the effect of salts on encystment be tested.

The problem of the effect of salts on encystment is a highly involved one. The presence of a selective-permeable membrane on the cell necessitates consideration of the migration of ions across the membrane, the antagonistic action of salts, and the change in hydrostatic pressure due to the presence of salts. Unless the composition of the salt solution is known, the effect of salts cannot be throughly understood. In view of this fact, an attempt was made to find a chemically known salt solution in which *Pleurotricha* will live and encyst.

A number of salt solutions are satisfactory for different organisms. One by one, these solutions were tried on *Pleurotricha*, but none proved satisfactory. These solutions are:

1. CHALKLEY Solution (for *Amoeba*).
2. HAHNERT Solution (for *Amoeba*).
3. KNOP Solution (for *Gonium*, etc.).
4. (A. W.) PETERS Solution (for *Stentor*).
5. (R. A.) PETERS Solution (for *Colpidium*).
6. PRINGSHEIM Solution (for various protozoa).
7. RINGER Solution (modified) (for tissue culture).
8. PACE Solution (for *Amoeba*).
9. MAST and PACE Solution (for *Chilomonas*).
10. Raffel Modified PRINGSHEIM Solution (for *Paramecium*).
11. BENECKE Solution (for Flagellates).
12. Modified RINGER Solution (for protozoa).
13. ZUMSTEIN Medium (for *Euglena*, etc.).

Of these solutions, some were more satisfactory than others, but disintegration of some individuals of *Pleurotricha* always occurred, indicating that they were, somehow, "toxic". By using the percentage of disintegration of the animal as a criterion, the relative toxicity of these solutions was ascertained. In general, the solutions containing organic chemicals were less toxic than those wich contained no organic substances and solutions with higher concentration of calcium salts were more favorable than those with higher concentration of sodium salts. This is in accord with HETHERINGTON's results (1932) on *Stentor* and with HAHNERT's on *Amoeba* (1932).

Two of these solutions, namely, PRINGSHEIM solution and A. W. PETERS solution, were found to be almost satisfactory. The percentage of disintegration of *Pleurotricha* in these two, varied from

2% to 30%, depending probably on the physiological condition of the individuals. Often when ten animals were placed in either one of these two solutions in a dish, some fifteen or sixteen animals were found the next day. It appeared that no disintegration occurred. But in all the tests with isolated single individuals, disintegration of some of the animals always occurred. This is not dependent upon the presence or the activity of bacteria, because experiments with sterile solutions yielded about the same percentage of disintegration as those with bacteria. It is not correlated with osmotic pressure because disintegration occurred in solutions of different concentrations within wide ranges. Animals taken from cultures of different ages manifested a similar percentage of disintegration, indicating that disintegration is not correlated with the age of the culture. By close observation, it was found that animals containing large vacuoles or a larger number of vacuoles, disintegrated in shorter time than those with smaller or with fewer vacuoles. However, selected animals with comparatively few small vacuoles did not show a reduction in the percentage of disintegration.

It is both interesting and difficult to understand that whenever complex organic materials such as hay-infusion or rye extract were added to the solutions listed above, disintegration did not occur. It appeared as though organic materials are essential for survival of this animal.

Failure to find a known inorganic salt solution satisfactory for pleurotricha banished the plan for making extensive studies on the effect of salts. However the effect of salts on encystment can be ascertained by adding them to a simple organic medium. This does not prove the effect of the action of individual salts on the animals, but does throw light on the effect of the various salts present. The balanced salt solution of A. W. Peters was chosen for this investigation. Solutions of different concentrations were prepared by adding these salts to the standard rye extract in place of water. A solution containing the salts in the concentration in the Peters solution was designated P, one containing the salts in twice this concentration 2 P, etc.

Experiments with and without sterile solutions were performed. The results obtained in all were practically the same. Those obtained in one are presented in table 14 (see p. 30).

The rates of encystment in concentrations 10 P and 5 P are not comparable with those in the other concentrations, because precipitation occurred with a consequent change of chemical composi-

Table 14.

Effect of Salts on the Rate of Encystment.

Concentration of salts	No. of individuals found	No. of cysts found	Av. % of encystment	Concentration of salts	No. of individuals found	No. of cysts found	Av. % of encystment
10 P	14	7		P/10	21	8	
	10	4			26	6	
	16	9	54.84		24	5	33.64
	9	8			17	9	
	13	6			19	8	
5 P	18	4		P/20	19	9	
	20	8			23	6	
	21	10	43.01		21	3	25.00
	18	7			25	4	
	16	11			20	5	
2 P	24	13		P/50	23	7	
	25	10			20	10	
	23	15	45.83		24	7	33.33
	24	8			16	8	
	24	9			25	4	
P	20	11		Control	18	10	
	25	7		No salts	23	7	
	23	14	44.83	added	23	6	33.03
	26	12			23	4	
	22	8			22	9	

tion. Table 14 shows that in concentrations $^1/_{10}$, $^1/_{20}$, and $^1/_{50}$ P, the rate of encystment was approximately the same as that in the control, but that in concentrations 2 P and P it was considerably higher. These differences in the rate of encystment are due to the effect of the action of the salts themselves and not due to the change of osmotic pressure of the medium, as will be demonstrated in the following experiment.

The facts that salts induce encystment and that urea and ammonia do not, indicate that it is the salts in the excretion products of organisms that are primarily responsible for the favorable action of these products on encystment.

k) Extraction of Water from the Organism.

It has been observed that during the process of encystment, a considerable amount of water is eliminated. It is also known that cysts can be obtained by drying a culture; therefore it is reasonable to think that encystment may be correlated with the extraction of water from the organism. In order to test this, the following experiment was performed:

Different concentrations of lactose solution were prepared by dissolving lactose in rye extract. The procedure of this experiment was similar to that of the foregoing experiments. The results are presented in table 15.

Table 15.

The Effect of Extraction of Water from the Organism on the Rate of Encystment.

Concentration of Lactose %	No. of individuals found	No. of cysts found	Av. % of encystment	Concentration of Lactose %	No. of individuals found	No. of cysts found	Av. % of encystment
	0	0			17	3	
	0	0			14	6	
5	0	0	0	0.03	13	4	30.87
	0	0			16	3	
	0	0			11	6	
	0	0			15	5	
	4	2			14	2	
2	0	0	40.00	0.01	14	4	30.00
	1	0			17	3	
	5	2			10	7	
	15	1			14	2	
	8	3			18	6	
1	11	5	29.83	0.001	12	5	35.29
	11	5			11	8	
	12	3			13	3	
	13	6			15	5	
	15	2			17	4	
0,1	11	5	30.56	Control,	13	3	33.83
	15	4		No Lac-	10	6	
	18	5		tose	13	5	

It will be observed from this table that the rate of encystment was no higher in the solution with lactose than in those without it. This indicates that extraction of water from the organism does not induce encystment. If this is true, it shows that encystment, which occurs so freely when natural ponds dry up, is due not to extraction of water from the organisms, but to increase in the concentration of salts.

4. Summary.

1. Deficiency of food in the medium induces encystment. Organisms containing little food material in the body encyst more readily than those containing much.

2. Rate of encystment is facilitated by moderate temperatures. Low temperatures do not prevent encystment; they merely retard it.

3. Rate of encystment is within wide ranges independent of hydrogen-ion concentration.

4. Rate of encystment increases with the age of infusion to a maximum, then declines and remains constant.

5. Excretion products of *Pleurotricha*, *Colpidium*, *Paramecium* and bacteria all facilitate encystment. This is probably due to the salt content of these products. It is not due to the urea or the ammonia content.

6. Rate of encystment increases to a maximum with the increase in concentration of salts. Further increase in concentration causes disintegration rather than encystment.

7. Urine and ammonia inhibit encystment.

8. Bacteria used as food lower the rate of encystment, but their excretion products facilitate encystment.

9. Crowding is not a direct factor in inducing encystment.

10. Extraction of water from the organism does not induce encystment; on the contrary it inhibits it.

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